Fibroblasts in atherosclerosis

Fibroblasts in atherosclerosis: heterogeneous and plastic participants

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Purpose of review
Fibroblasts are very heterogeneous and plastic cells in the vasculature. A growing interest in fibroblasts in healthy and atherosclerotic vasculature is observed, next to macrophages, endothelial cells, and smooth muscle cells (SMCs). In this review, we discuss fibroblast presence, heterogeneity, origin, and plasticity in health and atherosclerosis based on latest literature.

Recent findings
With help of single cell sequencing (SCS) techniques, we have gained more insight into presence and functions of fibroblasts in atherosclerosis. Next to SMCs, fibroblasts are extracellular matrix-producing cells abundant in the vasculature and involved in atherogenesis. Fibroblasts encompass a heterogeneous population and SCS data reveal several fibroblast clusters in healthy and atherosclerotic tissue with varying gene expression and function. Moreover, recent findings indicate interesting similarities between adventitial stem and/or progenitor cells and fibroblasts. Also, communication with inflammatory cells opens up a new therapeutic avenue.

Summary
Because of their highly plastic and heterogeneous nature, modulating fibroblast cell function and communication in the atherosclerotic vessel might be useful in battling atherosclerosis from within the plaque.

Keywords
atherosclerosis, fibroblast, mesenchymal cell

INTRODUCTION

Atherosclerosis and its clinical manifestations, for example myocardial infarction and stroke, are currently still the leading causes of death worldwide [1]. Atherosclerosis is characterized by lipid accumulation in the subendothelial space, intimal inflammation, smooth muscle cells (SMC) migration from the media to the outside of the newly formed plaque and ultimately plaque rupture [2]. Different cell types, including endothelial cells, macrophages, and SMCs, play prominent roles in this life-long process [3–5]. However, recent evidence suggests that an additional cell type, the fibroblast, is an important player in matrix production in atherosclerosis. Traditionally, fibroblasts are thought to arise from mesenchymal stem cells (MSCs) and are thus part of the mesenchymal cell category, also including pericytes and SMCs. In arterial injury, adventitial fibroblasts differentiate into activated fibroblasts (myofibroblasts) with de-novo alpha-smooth muscle actin (α-SMA) expression in response to proinflammatory cytokines, matrix remodeling, and transforming growth factor beta (TGF-β) signaling. Myofibroblasts have been implicated in extracellular matrix (ECM) production, proinflammatory cytokine, and matrix metalloproteinase (MMP) secretion and leukocyte recruitment [6–8]. However, these traditional views are being overturned by new insights and the advent of single cell sequencing (SCS), which will be discussed in this review.

In fact, the ability to acquire stem cell properties by upregulating markers such as stem cell antigen-1 (Sca-1) enables fibroblasts to be plastic and

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KEY POINTS

- Fibroblasts are ECM-producing cells abundant in the vasculature and involved in atherogenesis.
- Fibroblasts encompass a very heterogeneous population as indicated by SCS data revealing several fibroblast clusters in healthy and atherosclerotic tissue with varying gene expression and function.
- Fibroblast identity has been proposed for adventitial progenitor and/or stem cells and should be further investigated.

adaptable in numerous environmental situations [9▪,10]. Because of this heterogeneity and plasticity, currently used markers seem insufficient in unique identification of fibroblasts and/or covering the whole fibroblast population. Here, SCS will aid to find markers unique to fibroblasts. Indeed, SCS of healthy mouse brain confirmed the traditional marker platelet-derived growth factor alpha (Pdgfra) and yielded three new markers, decorin, lumican, and Mmp2 [11]. However, both lumican and decorin have been associated with other cell types involved in the advent of atherosclerosis [12▪▪,13]. This may suggest disease and/or organ specificity of markers to identify fibroblasts. The lack of a one-size-fits-all marker makes investigating their role in atherosclerosis development challenging. In this review, we aim to elucidate the functional role of fibroblasts in healthy and atherosclerotic vasculature by discussing fibroblast presence, heterogeneity, origin, and plasticity.

FIBROBLASTS IN HEALTHY VASCULATURE

The arterial wall consists of three layers. The inner intima is composed of an endothelial cell monolayer. The middle medial layer consists of SMCs embedded in ECM. Finally, the adventitia is the outer layer and is traditionally thought to harbor mesenchymal cells, that is fibroblasts, pericytes and SMCs, connective tissue, unmyelinated nerve fibers, resident leukocytes, small blood vessels with endothelial cells surrounded by mesenchymal cells, and several progenitor cells [8]. Multiple studies have shown the fibroblast's potential to extensively participate in organ homeostasis and repair mechanisms in response to stress [14–16]. The emergence of SCS has provided researchers the opportunity to study vascular cells in more depth. This technique has improved fibroblast annotation and revealed different subsets in multiple organs. Kalluri *et al.* [17▪▪] used abovementioned technique to investigate all three layers of the healthy murine aorta. The authors showed that SMCs comprise the largest cell population in the murine aorta (~40%), but surprisingly, also showed that fibroblasts make up for roughly 33% of aortic cells [17▪▪]. These fibroblasts consist of two subpopulations, with a phenotypic gradient rather than a rigid split between them. These fibroblasts are probably derived from the adventitia, although the authors removed perivascular fat – possibly including the adventitia. As their arterial wall location was not validated by immunohistochemistry or in-situ hybridization, a possible medial location for one or both subpopulations is yet to be confirmed. Furthermore, their function, embryonic origin, cellular progeny and fate are yet unknown. Gu *et al.* studied the adventitia of healthy murine aorta and shed more light on their function. They uncovered four mesenchymal populations, whose differential gene expression suggests functions in ECM organization, immune regulation and bone formation [18▪▪]. These data suggest fibroblast heterogeneity, already present in a healthy steady state.

FIBROBLASTS IN ATHEROSCLEROSIS

The classical dogma in atherogenesis entails migration of medial SMCs to the newly formed plaque, producing ECM components for fibrous cap formation [2]. This dogma has recently been challenged, as several groups have reported the presence of fibroblast-like cells in human atherosclerotic lesions [12▪▪,19]. Also, adventitial fibroblast-like cells have been functionally implicated in plaque ECM production [19,20]. Using $ApoE^{-/-}$ mice on a Western diet superimposed with chronic kidney disease, Kramann *et al.* [20] showed that a subset of adventitial MSC-like cells, expressing GLI family zinc finger 1 (Gli1), Sca1, and PDGFRβ, migrated into the media and neointima. Gli1+ cells contributed to calcification by differentiation into osteoblast-like cells. In contrast, Evrard *et al.* [19] reported decreased collagen and increased MMP expression in another subset of endothelial-derived, fibroblast-like cells expressing fibroblast activation protein (Fap) or fibroblast-specific protein 1 (Fsp1, S100a4 gene) in atherosclerosis, indicating a role in matrix degradation. In 2019, a key paper by Wirka *et al.* [12▪▪] employed SCS to assess cellular composition in atherosclerotic plaques from human coronary artery and mouse aorta, and identified two fibroblast clusters. Interestingly, Gli1, Fap, or Fsp1 were not among the top 100 differential genes in the two murine or human fibroblast subsets defined by Wirka, complicating the interpretation of the above reference studies and strongly suggesting

heterogeneity. Together, these studies suggest that fibroblast clusters identified in healthy and diseased tissue differ in functionality, possibly because of different origin and/or differentiation fate.

FIBROBLAST PLASTICITY, HETEROGENEITY, AND ORIGIN IN ATHEROSCLEROSIS

As described above, varying numbers of fibroblast clusters with corresponding differential gene sets have been identified in healthy and atherosclerotic tissue. Additionally, studies in other organs have shown that new fibroblast clusters can arise as a consequence of disease, further supporting plasticity and heterogeneity [15]. Heterogeneity makes it very difficult to identify the entire fibroblast population and a resulting lack of specific markers complicates fibroblast research. Common fibroblast markers, such as FAP, FSP1, and lumican are not specifically expressed by fibroblast-like cells only, and/or are not expressed by all fibroblasts [21–25]. Fibroblast heterogeneity may be a result of their various origins and enormous plasticity, all enhanced as a result of adaptation to disease. Here, we describe evidence to support that fibroblasts in atherosclerosis may also originate from SMCs and/or endothelial cells (Fig. 1). Also, we discuss adventitial stem and/or progenitor cells as a source of fibroblasts or possibly a subset of fibroblasts.

Smooth muscle cells origin

Wirka *et al.* studied SMC differentiation and their contribution to atherosclerosis *in vivo* combining SCS and a fluorescent myosin heavy chain 11 (Myh11) reporter strain for SMCs on an ApoE$^{-/-}$ background. In contrast to prevailing concepts of myofibroblast development from fibroblasts, they reported SMC differentiation into fibroblast-like, 'fibromyocyte' cells upon high-fat diet (HFD) [12▪▪]. In addition to two fibroblast and two SMC clusters in ApoE$^{-/-}$ mice on chow, a Myh11+ SMC cluster appeared and expanded with HFD feeding. This modulated SMC cluster showed decreased expression of SMC differentiation markers, and a clear transcriptional shift towards genes expressed by the fibroblast clusters, later confirmed in human coronary arteries [12▪▪]. Nevertheless, the cells were transcriptionally distinct from fibroblasts and displayed the Myh11-reporter. These data highlighted the benefits of fluorescent fate tracking and lead one to wonder whether these fibroblast-like cells have reached the end stage of their dedifferentiation or will dedifferentiate further into actual fibroblasts. Another question is, does the differentiation also occur the other way around? Comparison between the modulated SMC cluster and a myofibroblast population could be interesting to avoid off-target effects in future cell-specific targeting.

Endothelial origin

Another possible fibroblast source are endothelial cells, which can undergo endothelial-to-mesenchymal transitioning (EndMT). A review by Kovacic *et al.* [26▪] emphasized the functional importance of EndMT in both healthy and diseased vasculature. EndMT results in downregulation of endothelial-associated genes, such as cluster of differentiation 31 (CD31) or VE-cadherin, and upregulation of mesenchymal genes, such as α-SMA and FAP. These cells genetically present as mesenchymal cells and can execute mesenchymal functions like ECM production [26▪]. Evrard *et al.* [19] specifically showed that fibroblasts can arise through EndMT in atherosclerosis. Using a tamoxifen-inducible endothelial lineage-tracking system in ApoE$^{-/-}$ mice, they observed one-third of plaque cells positive for Fap were endothelial-derived after 8 weeks of HFD. The population expanded to nearly 50% in advanced atherosclerotic plaques [19]. They showed that EndMT is stimulated *in vitro* by severe hypoxia, TGF-β signaling, and oxidative stress, factors that are ubiquitous in atherosclerosis [19]. Oscillatory shear stress has also been identified as EndMT inducer in atherosclerosis [27]. Importantly, Evrard *et al.* [19] uncovered a relationship between the extent of EndMT and an unstable plaque phenotype in humans. Notably, the data should be interpreted with slight caution, as the markers used to identify fibroblasts are not unique [23,24]. Current SCS publications have not explicitly reported on EndMT, either because it was unstudied or possibly because of lack of sufficient cells to model transitions. However, the reported two fibroblast human subsets could include EndMT-derived cells. The top 100 differential genes do not include endothelial markers, yet this does not exclude low marker expression [12▪▪]. Hence, SCS using endothelial cell reporter strains are yet to fully confirm these findings. In addition, the functional differences between both EndMT-derived and SMC-derived fibroblast (-like) cells and their exact contribution to atherosclerosis remain to be elucidated.

Adventitial stem and/or progenitor cells

Fibroblasts have been suggested to originate from a pool of adventitial stem and/or progenitor cells. However, the identity of these cells is a point of discussion, as fibroblasts also have the ability to re-acquire stem cell properties by upregulating markers such as Sca-1 [9▪,10,20,28]. Additionally, MSCs and fibroblasts are

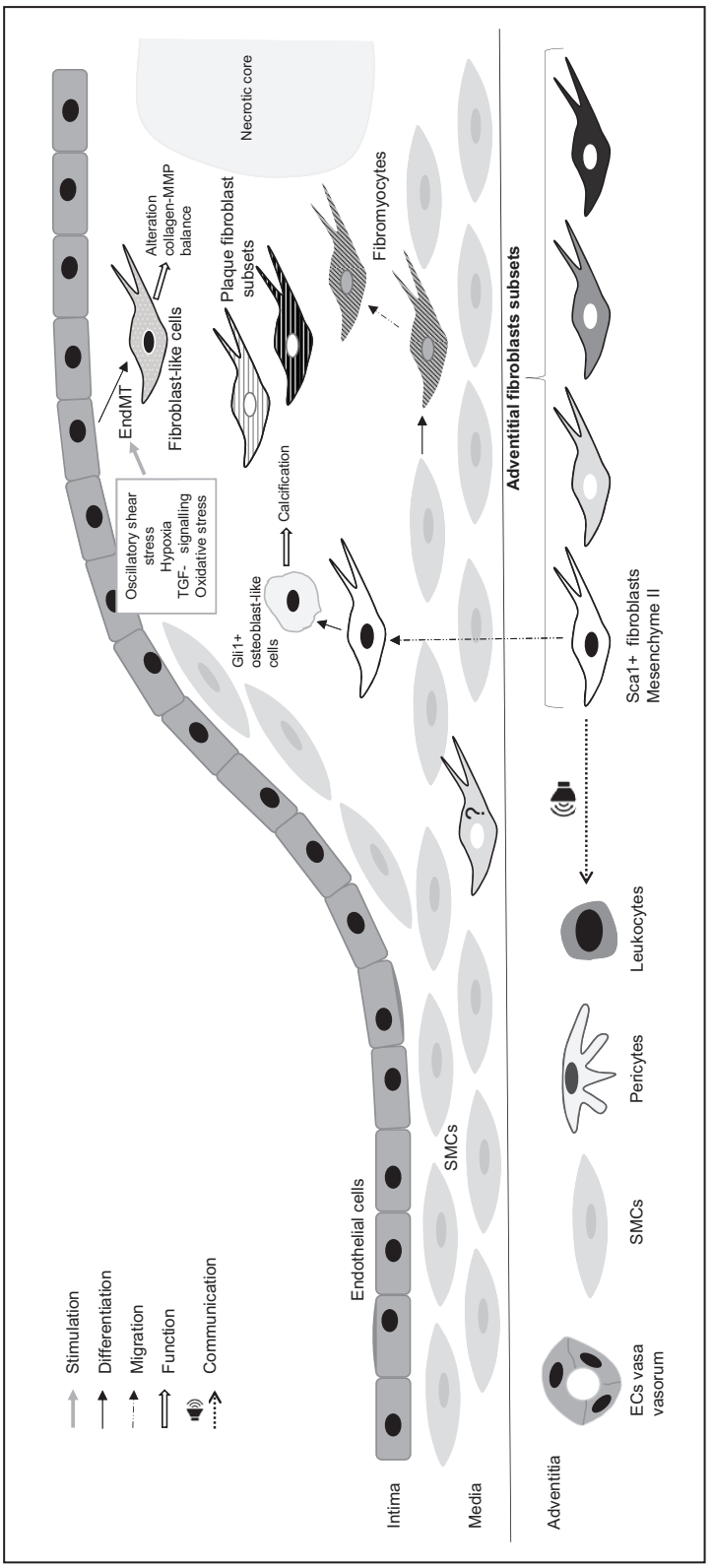

FIGURE 1. Presence and origin of fibroblasts in atherosclerosis and their suggested contributions. Four adventitial fibroblasts subsets have been discovered using single cell sequencing (SCS). Sca1+ fibroblasts may contribute to atherosclerosis by migrating into the neointima. Gli1+, Sca1+ adventitial stem/progenitor cells have been shown to differentiate into osteoblast-like cells and hereby contribute to plaque calcification. Two fibroblast subsets have been identified with SCS, while prior studies showed that fibroblast(-like) cells in atherosclerosis can originate from medial smooth muscle cells (SMCs), called fibromyocytes, and from endothelial cells through endothelial-to-mesenchymal transition (EndMT). It is unclear whether these are similar to or distinct from the two fibroblast subsets discovered by SCS. ? indicates possible medial localization of fibroblasts.

morphologically similar and expression of MSC surface markers, such as CD105, CD73, and CD90, has been observed on fibroblasts. Vice versa, MSC expression of common fibroblast markers, that is vimentin and fibroblast surface protein (FSP) has been reported [9▪]. Similar to MSCs, fibroblasts seem capable of differentiation into adipogenic, osteoblastogenic, and chondrogenic lineages [9▪]. These insights might suggest that adventitial MSCs and Sca-1+ progenitor cells, previously identified and studied by many groups, are in fact fibroblasts. Indeed, a recent paper by Ni *et al.* shows that 10% of c-Kit+ cells was positive for the fibroblast marker PDGFRA in healthy C57Bl6 aorta [29▪]. Their findings were confirmed using an inducible Cre model, labeling c-Kit+ cells with TdTomato, showing ~20% overlap between PDGFRA and c-Kit [29▪]. Moreover, Tang *et al.* [21,30▪] also reported that 40% of adventitial Sca-1+ cells with progenitor properties coexpressed PDGFRA. These Sca-1+/PDGFRA+, progenitor-like cells generated new medial SMCs after severe artery injury [30▪]. Similar to other recent studies that assessed vasculature cell populations by SCS, Gu *et al.* [18▪▪] did not annotate mesenchymal clusters in adventitia of ApoE$^{-/-}$ and wildtype aortas as stem or progenitor cells. Yet, one of the four identified mesenchyme clusters showed high Sca-1+ expression, indicating stem cell properties of this cluster [18▪▪]. The distinction between true adventitial stem and/or progenitor cells and fibroblasts may thus be smaller than previously assumed, and expression of Sca1+ indicative of fibroblast plasticity.

Together, these data suggest that fibroblasts show an even greater plasticity than previously thought. Cell transitioning of fibroblasts into other cell types and vice versa seems common and extensive in atherosclerosis. Whether all currently identified adventitial stem and/or progenitor cells are really adventitial fibroblasts and vice versa is an important remaining question to be resolved using reliable fibroblast reporter models. Based on this concept, another question is whether the fibroblast is an end-stage cell or merely a collection of heterogeneous 'in between' cells, actively transitioning between different cell types, or a combination of the two. It would be interesting to study if the acquisition of stem cell-like properties by fibroblasts occurs through dedifferentiation. Assessing the differentiation capacity of the distinct fibroblast clusters into other cell types could also shine some light on this discussion.

FIBROBLAST CELL–CELL COMMUNICATION AND ITS THERAPEUTIC POTENTIAL

In addition to heterogeneity and function of fibroblasts in the natural development of atherosclerosis, these cells could possibly be used as a new therapeutic approach based on their effect on surrounding inflammatory cells. A proinflammatory role of mesenchyme clusters through increased intercellular communication with inflammatory macrophages has been computationally predicted in ApoE$^{-/-}$ adventitia by Gu *et al.* [18▪▪]. A recent paper by Gorabi *et al.* [31▪] also reviewed the possibility of using MSCs as treatment for atherosclerosis by modulating inflammation. Multiple studies discussed in this review showed a marked anti-inflammatory effect in murine atherosclerosis by decreased proinflammatory cytokines and nuclear factor-kappa B signaling after bone marrow MSC administration. MSC therapy has been studied in clinical trials for diseases such as heart disease, cancer and peripheral artery disease, but not atherosclerosis. It is considered a promising future treatment option, but at the same time its safety and efficacy are questioned. Knowledge regarding precise in-vivo mechanisms of action is still lacking and inconsistent results are observed because of cellular heterogeneity of MSCs and a lack of specific markers [32]. Donor characteristics, culture conditions, method and location of delivery, and host receptibility are all factors that can influence MSC therapy efficacy and efficiency [31▪,32,33]. Moreover, risks of malignant transformation and protumorigenic effects of MSCs have been reported. Thus, extensive additional research into improving efficiency and efficacy of MSC therapy is required before considering this a new therapy option.

CONCLUSION AND FUTURE RESEARCH

The present review shows that in contrast to the assumptions the classical dogmas contain, next to SMCs, fibroblasts are ECM-producing cells abundant in the vasculature and involved in atherosclerosis. Fibroblasts comprise a very heterogeneous population because of different cellular origins and an extensive repertoire of possible cell transitions. The origin and fate of fibroblasts in atherosclerotic plaques remains to be elucidated. Because of their heterogeneity, there is a lack of specific markers that encompass the entire population making it difficult to study fibroblast (sub)populations in atherosclerosis. Recent comparisons between fibroblasts and adventitial stem and/or progenitor cells indicate similarities between these cells. Moreover, recent SCS data did not identify any adventitial stem and/or progenitor cell clusters, supporting fibroblast identity of these cells. SCS data did identify multiple fibroblast clusters with differential gene expression and functionality per cluster in healthy and atherosclerotic tissue. Further research into subpopulations of fibroblasts and their different functions is needed to identify specific

markers per subpopulation and to determine the contribution of each subpopulation to atherosclerosis. The emergence of SCS provides opportunities to find answers to the remaining questions in an unbiased way. In the future, modulating fibroblast cell communication in atherosclerotic vessels could be useful in battling atherosclerosis from within the plaque.

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Conflicts of interest

There are no conflicts of interest.

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